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## **Th-17 Cells Infiltrate the Liver in Human Biliary Atresia and are Related to Prognosis**

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**Footnote Page****Contact Information**

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**List of Abbreviations**

Include the expansions and list in the order of their mention in the paper.

Th-1, Th-2 – T helper 1 and 2

Tregs – regulatory T cells

CMV IgM – cytomegalovirus immunoglobulin M

BASM – Biliary atresia splenic malformation syndrome

AST - aspartate aminotransferase

ALP - alkaline phosphatase

GGT -  $\gamma$ -glutamyl transferase (GGT)

PCR –polymerase chain reaction

RRV – Rhesus rotavirus

CXCL - chemokine

CMA –cellular adhesion molecule

ICAM – intercellular adhesion molecule

VCAM – vascular adhesion molecule

IL- interleukin

NK cell - Natural Killer cell

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- MD, NH, GMV & DV originated the study. RH, MH and AQ carried out the immunohistochemical and histological analysis and collated the clinical data. MD & RH wrote the first draft. All authors agree with the contents of the final submitted version.

## Abstract

**Background:** Biliary atresia (BA), a cholangiopathy of unknown aetiology affecting infants, is frequently associated with intrahepatic mononuclear cell infiltrate and an abnormal reaction to viral exposure has been hypothesised in some cases.

**AIM:** to investigate the nature of the CD4+ hepatic infiltrate in defined clinical variants of BA by quantifying the various inflammatory cell components.

**Methods:** Liver biopsies of BA infants obtained at Kasai portoenterostomy (KPE) were stained immunohistochemically using monoclonal antibodies to Tbet, GATA-3, FOXP3 and IL-17, identifying Th-1, Th-2, Tregs and Th-17 cells respectively. T cells were counted using a graticule technique. Data are reported as median (range) of cells per high-power-field (x400) and compared using non-parametric statistical tests with  $P \leq 0.05$  regarded as significant.

**Main Results:** Liver biopsies from BA ( $n=37$ ) and age-matched cholestatic controls ( $n=12$ ) were used. BA infants were divided into three groups: cytomegalovirus IgM+ve (CMV;  $n=9$ ); BA splenic malformation (BASM;  $n=9$ ) and isolated BA (IBA;  $n=19$ ). All T-cell subsets were present in the portal tracts, with an overrepresentation of Th-1 ( $P<0.001$ ) and Th-17 ( $P<0.03$ ), but not Th-2 ( $P=0.94$ ) or Tregs ( $P=0.15$ ), compared to controls. Th-1 cells predominated in the CMV group; [18 (7-37) vs. 3 (0 – 14) (BASM) and vs. 5 (3 – 23) (IBA);  $P<0.01$  both], while no sub-group differences were seen for Th17 cells. The degree of Th-1 cell infiltrate inversely correlated with platelet count ( $r_s=-0.49$ ;  $P<0.01$ ).

Th17 cells were fewer [6(2–11) vs. 11(8-20);  $P=0.02$ ] in infants who cleared their jaundice ( $n=15$ ,  $<20 \mu\text{mol/L}$ ) although this did not translate to improved native liver survival ( $P=0.17$ ).

**Conclusion:** Th-17 cells infiltrate the liver in BA and are associated with a worse surgical outcome; a Th-1 profile predominates in CMV-associated BA.

## Introduction

Biliary Atresia (BA) is an occlusive pan-ductular cholangiopathy presenting in neonatal life with an incidence in Europe ranging from 1 in 17-20,000 (1,2). Characteristically, there is cholestasis with a progressive inflammatory infiltrate in most (but not all) cases and an early-onset of liver fibrosis leading ultimately to cirrhosis and end-stage liver failure within six months if untreated (3).

A single uniform pathogenic mechanism appears unlikely since there is much clinical evidence for aetiological heterogeneity with BA being simply the final common phenotype. There are infants with clear evidence of a **developmental** defect, either because the BA is one of a range of disparate anomalies such as situs inversus, polysplenia and venous anomalies [i.e. Biliary Atresia Splenic Malformation syndrome (BASM) (5), or because the bile duct abnormality has structural changes making it detectable from the 16<sup>th</sup> week of gestation (i.e. cystic biliary stricture) (6). More common are other infants, labelled as **Isolated BA**, who still may have primary developmental bile duct failure, but for which there is no clinical evidence. Alternatively, infants may have normal bile ducts at birth, which are then damaged secondarily, perhaps by perinatal viral infection or immune-mediated injury (7).

A purported relationship to viral infection has been suggested for at least 30 years although substantive clinical evidence has been lacking. A wide range of enteric viruses has been implicated including REOvirus and cytomegalovirus (CMV) but clinical evidence of exposure and its relevance is conflicting whether derived from serological studies (7,8,9) or more directly from PCR studies on liver biopsies to detect viral nucleic acids (10).

One of the characteristics of the liver in BA is abnormal over-expression of Class II HLA and inflammatory adhesion molecules (e.g. ICAM, VCAM, e-selectin) on bile duct and vascular epithelium in isolated BA together with a marked inflammatory lymphocytic infiltrate (11,12). Our initial immunohistochemical studies showed this to be predominantly CD4+ T cells and CD56+ NK cells with evidence of activation (CD25) and proliferation (CD71)(13). Later studies characterized this as a Th1 cell mediated portal tract inflammation (through production of IL-2, IFN- $\gamma$  and TNF- $\alpha$ ) (6). Such Th1 cell subsets were shown to be oligoclonal suggesting that the T cells in BA proliferate in response to a specific antigen, such as viral proteins, or even 'self' bile duct epithelial proteins (14). Later studies have concentrated on the alternative effector arm of the immune response involving CD8+ and NK cells. Thus, Guo et al. isolated CD8+ T cells and CD56+ NK cells from BA livers and showed marked

increases in their activation cytokine profile together with the evidence of cholangiocyte lysis in an *in-vitro* model (15).

Recent advances in CD4+ lymphocyte genealogy has defined two further subsets - regulatory T-cells (Tregs) and Th-17 cells (defined by secretion of the cytokine IL-17). The former are known to be important in the prevention of autoimmune diseases by maintaining immunological self-tolerance and experimental studies in the RRV murine model of BA have shown that lack of Tregs in the first three days of life allows an unopposed viral infection to cause bile duct inflammation and occlusion (16). Th-17 cells, by contrast, have a destructive role in autoimmune biliary diseases such as primary biliary cirrhosis (17) and autoimmune hepatitis (18).

The aim of this study was to investigate the nature of the CD4+ inflammatory infiltrate in BA livers with quantification of the various inflammatory cell components and to try and relate this with the defined clinical variants.

## **Materials and Methods**

Kings College Hospital is the largest specialist unit for paediatric hepatobiliary disease in the UK and a designated referral centre for infants with BA. We identified from a prospectively-maintained database four distinct clinical groups according to diagnosis and their age at Kasai portoenterostomy (KPE). These included infants with histologically-proven BA who were: (i) CMV IgM+ve at the time of diagnosis; (ii) BASM; (iii) isolated BA, < 60 days at time of KPE or (iv) isolated BA >60 days at time of KPE. Surgery was performed using a published technique by a single surgeon (MD). All had had wedge liver biopsies taken from the right lobe at the end of the operation and stored in a designated tissue bank.

Core liver biopsy material was obtained from age-matched infants with proven  $\alpha$ -1 antitrypsin deficiency and Alagille syndrome for use as neonatal cholestatic controls.

Biopsies were initially fixed in 10% formalin at room temperature for between one and 24 hours. They were then dehydrated in graded alcohol and xylene solutions before being processed in a Tissue-Tek® VIP® 5 Vacuum Infiltration Processor (Sakura Finetek USA, Torrance, CA). Finally they were embedded in paraffin wax. Sections were cut at 4µm thickness.

**Immunohistochemistry.** Two different immunohistochemical techniques were used (19, 20) to identify Tbet, GATA3, FOXP3 and IL-17.

The Novocastra™ Novolink™ Polymer Detection System (Leica Microsystems, Newcastle, UK) was used to look for the presence of Th1, Th2 and Tregs using antibodies to Tbet, GATA-3 and FOXP3. Paraffin sections were deparaffinised in xylene before boiling in a water bath containing Tris-EDTA buffer at pH 9 for antigen retrieval. Endogenous peroxidase activity was neutralised with application of the Novocastra™ Peroxidase Block (3% hydrogen peroxide). To avoid diffuse, non-specific background staining, Novocastra™ Protein Block was then applied. The sections were then incubated with mouse monoclonal primary antibody (to Tbet, GATA-3 and FoxP3) for one hour at room temperature after which time the Post-Primary block was applied for 30 minutes. Following this the slides were incubated with the secondary antibody, Novolink™ polymer (anti-mouse IgG). Peroxidase activity was developed using a mixture of DAB substrate solution and produced a visible brown precipitate at the antigen site. Biopsies were then washed and counter-stained with haematoxylin.

An avidin-biotin immunoperoxidase technique was used to detect IL-17. Paraffin sections were deparaffinised in xylene and then re-hydrated in ethanol. The antigens were unmasked by boiling the sections in sodium citrate buffer at pH 6. Endogenous peroxidase was neutralised by incubating with Novocastra™ peroxidase block (3% hydrogen peroxide). Following this, the biopsies were incubated for 10 minutes with 10% horse serum to inhibit non-specific background staining. After washing, avidin and then biotin blocking reagents were applied for 15 minutes each. The sections were incubated with a polyclonal goat anti-human IL-17 antibody (R&D Systems®, Minneapolis, USA) for one hour at room temperature at a 1:30 concentration. The sections were then incubated with biotin conjugated secondary antibody (horse anti-goat IgG, diluted 1:50) for 30 minutes. Bound antibody was visualised by incubating with Avidin: Biotinylated Peroxidase Complex (Vectastain® Elite® ABC Kit, Vector Laboratories Inc, California, USA). Peroxidase activity was then developed using a mixture of DAB substrate solution. The slides were then counter-stained with haematoxylin.

### **Cell Quantification**

Immunostained sections were evaluated using an Olympus BX51® light microscope (Olympus Microscopy, Southend-on-Sea, UK). Cell counting was performed according to a previously published technique (21, 22) using an 'eyepiece graticule' attachment (0.25mm<sup>2</sup>) to superimpose a cell



counting grid (Datasights, Ltd., Middlesex, UK) onto a magnified slide image. Ten randomly chosen portal tracts and ten lobules were counted by two observers (RH, AQ). This was then expressed as the median count / grid [at high power field (hpf) magnification x400].

Clinical details including preoperative liver biochemistry [total bilirubin, aspartate aminotransferase (AST), alkaline phosphatase (ALP),  $\gamma$ -glutamyl transferase (GGT) and platelet count] and clinical outcome were obtained from the database. Outcome was defined by clearance of jaundice (bilirubin  $<20 \mu\text{mol/L}$ ) within 6 months of KPE and Kaplan-Meier survival curves constructed to illustrate native liver survival.

Institutional and ethical approval was obtained for the study (REC 11/LO/0430).

Data are presented as medians and range, unless otherwise indicated, and intergroup comparison evaluated using non-parametric ANOVA (Kruskal-Wallis test) with post-hoc analysis by Mann-Whitney U test or Wilcoxon matched pairs as appropriate. Correlation was investigated using the Spearman rank correlation coefficient. Survival data were compared using the log-rank test. Statistical analyses were performed using GraphPad Prism® (GraphPad Software, Inc. Version 5. La Jolla, California, USA). *P* values  $<0.05$  were considered significant.

## Results

Liver biopsies from 49 infants were evaluated and scored (BA,  $n = 37$ ; alpha-1-antitrypsin,  $n = 6$ ; Alagille syndrome,  $n = 6$ ). Those with BA had all undergone a KPE between Sept. 2004 and Nov. 2010 and were divided into (i) CMV IgM+ve ( $n = 9$ ), (ii) BASM ( $n = 9$ ), (iii) isolated BA,  $< 60$  days at time of KPE ( $n = 9$ ) and (iv) isolated BA  $>60$  days at time of KPE ( $n = 10$ ). For comparative purposes groups (iii) and (iv) were usually combined as “isolated BA” (IBA) ( $n = 19$ ).

Median (range) age at liver biopsy in the control groups was 57 (21-96) days. Median age at KPE was 62 (36 - 141) days (group i); 63 (44 – 94) days (group ii); 42 (36 – 47) days (group iii) and 67 (60 – 70) days (group iv) in the BA sub-groups.

All T cell subsets were present principally in the portal tracts of infants with BA but in varying quantities (*Table 1*). Broadly, these consisted of Th-1 [7 (0 – 37) cells/hpf] and Th-17 [9 (0 – 105)

cells/hpf] with very few Th-2 [0 (0 – 2) cells/hpf] and Tregs [1 (0 – 7) cells/hpf] cells (**Figure 1**). There was no significant correlation between the two major T cell subsets ( $r_s = -0.33$ ,  $P = 0.25$ ).

Successful staining of control material was more problematic (except for Th-17 cells) with only small numbers in each group. Nevertheless there were significantly more Th-17 and Th-1 cells in BA infants than cholestatic controls (e.g. portal tracts: 9 (0 – 105) vs. 1.5 (0 – 5) cells / hpf;  $P = 0.0002$  and 7 (0 – 37) vs. 2 (0 – 11) cells/hpf;  $P = 0.03$  respectively).

### **Th-1 Lymphocytes.**

The Th-1 cells (Tbet+) were found in significantly higher concentrations in the portal tracts compared to the lobules of the BA livers [7 (0–37) vs. 3 (0–35) cells/hpf;  $P = 0.001$ ] (**Figure 2**). There were quantitative differences between the three sub-groups (KW test,  $P = 0.0008$ ). Thus, there were significantly more portal tract Th-1 cells in the CMV group than in both IBA [18 (7–37) vs. 5 (3–23) cells/hpf;  $P=0.002$ ] and BASM [3 (0–14) cells/hpf;  $P=0.002$ ]. There was no significant difference between the BASM and IBA groups [3 (0–14) vs. 5 (3–23) cells/hpf;  $P = 0.08$ ]. (**Figure 3a**).

### **Th-2 Lymphocytes.**

There were very few Th-2 (GATA3+) cells seen in any of the biopsies with little difference between portal tract and lobule; and no difference between BA and controls ( $P = 0.94$ ) nor within the BA sub-groups ( $P = 0.8$ ).

### **Regulatory T Lymphocytes.**

Again, there were very few Tregs (FOXP3+) cells in any of the biopsies (**Figure 2**). These were confined to the portal tracts rather than the lobules of BA livers [1 (0 – 7) vs. 0 (0 – 1) cells/hpf;  $P = 0.004$ ]. There was no difference between BA and control livers ([1 (0 – 7) vs. 0 (0–1);  $P = 0.07$ ] nor within the BA sub-sets ( $P = 0.49$ ).

### **Th-17 Lymphocytes.**

Th-17 cells were heavily concentrated within the portal tracts as compared to the lobule [9 (0 – 105) vs. 0 (0 – 4) cells/hpf;  $P<0.0001$ ] (**Figure 2**). There were no significant quantitative differences between the three BA sub-groups (KW test  $P = 0.52$ ) (**Figure 3b**).

We found no quantitative difference between the two isolated BA groups differentiated by age at KPE (iii and iv) for the two major T cell sub-groups [Th-1 ( $P = 0.45$ ) and Th-17 ( $P = 0.31$ )].

### ***Liver biochemistry and haematology.***

There was no correlation between portal tract Th-1 or Th-17 cell counts in BA livers and their pre-operative bilirubin, AST or  $\gamma$ -GT (*Table 2*). There was a modest correlation between the aspartate aminotransferase platelet ratio index (APRI) (28) and Th-1 ( $r_s = 0.32$ ,  $P = 0.05$ ).

### ***Relationship with outcome.***

Overall 15 (41%) infants cleared their jaundice (bilirubin  $<20 \mu\text{mol/L}$ ) by six months post-KPE. These infants had lower portal tract Th-17 counts than those who did not [6 (0 – 15) vs. 11.5 (0 – 105) cells/hpf;  $P = 0.008$ ] (**Figure 4a**). In contrast, there was no difference in Th-1 counts [7 (2–37) vs. 7 (0 – 33) cells/hpf,  $P = 0.49$ ] (**Figure 4b**).

There were 5 deaths in the series and 21 patients had a liver transplant at a median age of 15 (5 – 30) months leaving 11 who are alive with their native liver. **Figure 5** illustrates the native liver survival curve of the BA children divided according the degree of Th-17 infiltration. The difference did not reach statistical significance ( $P = 0.17$ ).

## **Discussion**

The heterogeneity and rarity of human BA has limited interpretation of studies aimed at defining the nature of the inflammatory response, believed to be a fundamental characteristic of its pathogenesis. Most detailed studies have been based upon a single experimental model, the RRV-Balb/c mouse model, whose actual relationship to events in humans is arguable.

We have investigated the distribution of the T cell portal tract infiltrate in subgroups of BA likely to have different aetiology, selecting infants with consistent clinical features enabling appropriate categorization. This has allowed reasonable numbers of relatively uncommon clinical variants (e.g. BASM) to be studied. We have also defined a group of infants likely to have had a viral contribution to their pathogenesis by the presence of CMV IgM antibodies.

Our previous immunohistochemical study shows that the predominant cellular immune response in the majority (but not all) of infants with BA consists of an activated CD4 and NK cell portal tract infiltrate. The present study shows a Th-1 and Th-17 predominance quantitatively and for the first time shows a marked relationship of the former with CMV IgM+ve associated BA.

Several hepatotropic viruses have been incriminated at some time in BA with most studies concentrating on a single virus. By contrast, Rauschenfels et al. (23) investigated a panel of viruses in German infants, identifying evidence of viral RNA/DNA in liver biopsies in about 45%, with the most common being REOVirus followed by CMV. Viral RNA/DNA positivity did not make any difference to survival and outcome in a later published follow-up series (24), leading the authors to question whether this had any clinical importance. Most recent clinical studies have concentrated on the putative role of CMV rather than REOVirus in BA. The proportion of CMV IgM +ve infants in our clinical series is ~ 10% (unpublished observation) and is similar to the German series. These infants are older at presentation, have more severe biochemical indices of liver damage (AST, APRI) and a different histological appearance of the liver (broadly inflammatory rather than cholestatic) (unpublished observation). Brindley et al. (25) studied liver memory T cell response to CMV proteins and found that 56% of BA infants had significant increases in IFN- $\gamma$ -producing liver T cells when exposed to CMV proteins suggesting perinatal CMV infection. There is, however, a marked geographical variation in CMV +ve BA with only about 10% of all BA infants affected in European series (23) compared to 30-50% in those recently reported from Brazil (26) and China (27).

We report here that a major component of the inflammatory infiltrate in BA consists of Th-17 (IL-17+) cells. The presence of Th-1 and Th-17 cells appears to be independent with little correlation between the two subsets. There is also no correlation with conventional biochemical indices of liver damage (AST) or bile duct obstruction (bilirubin,  $\gamma$ -GT) and Th-1 or Th-17 tissue lymphocytes, though we have found a modest relationship between Th1 counts and a novel surrogate marker of liver fibrosis, the APRI (28). Th-17 cells have been linked to chronic inflammation in primary biliary cirrhosis (17, 29), which is believed to have an autoimmune pathogenesis, and in chronic viral hepatitis. In these conditions, Th-17 cells are reported to concentrate at the interface of inflamed portal tracts and damaged interlobular bile ducts. Th-17 cells secrete a range of proinflammatory cytokines such as IL-23, IL-6, IL-8 and IL-1 and chemokines such as CXCL (CXC-chemokine ligand)-1, CXCL2, CXCL3 etc.

Previous work on Th-17 cells and human BA has been limited to a single Chinese study (30). In this, there appeared to be a fourfold increase in the proportion of peripheral Th-17 cells in infants with isolated BA compared with healthy controls (e.g. intestinal atresia) and more Th-17 cells were identified in the portal tracts of BA livers than in healthy control. Furthermore qRT-PCR analysis found that there were 5-6 fold differences in the Th-17 specific transcription factor ROR- $\gamma$ t and IL-

17a in BA compared to healthy controls. Unlike our own study, no attempt was made to distinguish between different underlying causes of BA.

Regulatory T cells (CD4+CD25+FoxP3+) suppress the immune process and have been used for the treatment of experimental models of inflammatory/autoimmune disease such as colitis (31) and autoimmune hepatitis (32). The absence of Tregs in the early neonatal period has been suggested as one mechanism whereby there is an unrestricted immune response directed against targeted cholangiocytes (33). This hypothesis has been largely based on evidence from the Balb/c mouse model where adoptive transfer and supplementation of the Treg subset abrogated the biliary damage and Treg neutralization prolonged the neonatal window of susceptibility to Rhesus Rotavirus, possibly by inhibiting CD86 expression on myeloid dendritic cells (34). Tregs have been studied also in the human disease: Brindley et al. (25) have reported differences in peripheral FOXP3+ numbers and proportion between 21 infants with isolated BA (and specifically those with CMV antibodies and controls. Yang et al. (30) recently showed a two-fold increase in FOXP3+ liver infiltration in BA, but a decreased proportion among the peripheral blood mononuclear cells compared to healthy controls. Our quantification study however, did not show any difference in infiltrating hepatic Treg numbers between pathological or control groups, though demonstrating differences would have been difficult because of the paucity of Tregs in all samples.

A role for the Th-2 pathway is more controversial, though recent murine studies have highlighted a Th-2 driven inflammatory response (particularly via IL-13) (35) and B-cell autoimmunity (36) with the presence of autoantibodies against  $\alpha$ -enolase, an enzyme involved in glycolysis and expressed in both bile duct epithelia and hepatocytes, in BA. Autoantibodies to  $\alpha$ -enolase have been described in patients with BA, suggesting a role for humoral autoimmunity perhaps as a secondary response targeting bile duct epithelia (36). In contrast to these results, we could not confirm the presence of a Th-2 infiltrate in our clinical series.

An unknown proportion of infants with BA have a true developmental biliary defect. Most obviously these are infants with other congenital abnormalities such as BASM, but there are other scenarios, such as cystic BA, where a biliary pathology can be detected on the maternal ultrasound during the 2<sup>nd</sup> trimester (5). We have previously reported a series of 3 neonates with BASM where the liver at the time of birth appeared, and was histologically, entirely normal (37). Thus even in patients with BA where we can be almost certain that the bile duct defect had occurred during the 1<sup>st</sup> trimester (in common with the other components of the syndrome), there was no histologically evident liver

damage and certainly no fibrosis. It also seems reasonable to infer that in these any inflammatory process might be less marked or indeed entirely absent. Nonetheless, we could not find any difference in the nature of the infiltrate, in cell absolute numbers or in the proportions of the two major T-cell subsets, Th-1 and Th-17, in our infants with typical BASM features. This suggests that also in BASM there is the same inflammatory process but that its induction and persistence may be due to different mechanisms, such as disruption of biliary ductules and perhaps leakage of cytotoxic bile acids outside of the lumen.

The key to outcome in BA is clearance of jaundice. Interestingly, we have found a two-fold difference in Th-17 counts between patients who failed to achieve a normal bilirubin level post-KPE and those who did. However, just as the cause of biliary atresia can be heterogenous, so the response to the KPE is also multifactorial and caution should be exerted in the interpretation of a single observation, particularly when cellular in nature (13, 38,29). In our previous immunohistochemical studies we reported that a liver infiltrate characterized by lower numbers of macrophages (CD68+) at the time of surgery was correlated to better outcomes at one year in 28 children with BA (13). A similar observation was made by Kobayashi et al. in their study of 15 infants (40). Similarly, we showed (38) that raised sVCAM levels at the time of KPE were associated with a poorer outcome in a study of 61 infants and that post-operative elevation of a range of cell adhesion molecules and cytokines such (IL-2, interferon- $\gamma$ , IL-4, IL-10, TNF $\alpha$  and sICAM-1) was associated with an increased need for early transplantation (39).

In conclusion, in this selected study of infants with different types of biliary atresia we have shown largely similar inflammatory liver infiltrates consisting of two principal T cell subsets, Th-1 and Th-17. The observation of a different inflammatory tissue composition, mainly composed of Th1 cells, in those children who were CMV-IgM positive provides circumstantial evidence that CMV-associated BA may represent a distinct entity.

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## FIGURE LEGENDS

**Figure 1:** Quantitation of T cell subsets in biliary atresia (n = 37) (mean +SEM).

**Figure 2:** Th-1 (upper), Th-17 (middle ), Treg (lower) and Th-2 (lower) infiltration in infants with biliary atresia.

**Upper Row** A (x20) B (x40) Th-1 cells scattered throughout portal tracts and liver of XX day infant with isolated BA.

**Middle Row** A (x10) B (x20) Th-17 cells heavily concentrated in the portal tracts of a liver from XX day old infant with isolated BA.

**Lower Row** A (x40) B (x40). **A** Shows very few Treg (FOXP3+) cells in liver from XX day infant with isolated BA. **B** shows very few Th2 cells in a BA patient again at 40x magnification.

**Figures 3a & b:**

T cell subset quantification [(a) Th1 and (b) Th17] according to perceived aetiology: CMV IgM+ve BA (n = 9); BASM (n = 9); isolated BA (n = 19).

**Figure 4 (a & b):**

Clearance of jaundice and portal tract T cell subset [Th-17 (a) and Th-1 (b)]

**Figure 5:**

Native liver survival curve - High ( $\geq 9$  / hpf) *versus* Low ( $<9$  hpf) Th-17 portal tract infiltration.

## TABLES

Table 1: Quantification of T cell subsets in portal areas of liver

<b><i>Biliary Atresia Groups</i></b>					
	<b>N</b>	<b>Th-1 (Tbet)</b>	<b>Th-2 (GATA)</b>	<b>Th-17 (IL-17)</b>	<b>Tregs (FOXP3)</b>
<b>CMV IgM+</b>	9	18 (7 – 37)*	0 (0 – 0)	6.5 (0 – 26)	1 (0 – 3)
<b>BASM</b>	9	3 (0 – 14)	0 (0 – 0.5)	8 (0 – 105)	0 (0 – 2)
<b>IBA (all ages)</b>	19	5 (3 – 23)	0 (0 – 2)	9 (0 – 30)	0 (0 – 7)
<b>IBA &lt;60 days</b>	9	5 (3 – 23)	0 (0 – 2)	9 (2 – 30)	0 (0 – 1)
<b>IBA &gt;60 days</b>	10	5 (3 – 16)	0 (0 – 0.5)	10 (0 – 25)	1 (0 – 7)
<b><i>Control Groups</i></b>					
<b>Alpha-1 Antitrypsin</b>	6	8, 10	0,3	2 (1 – 5)	0, 1
<b>Alagille syndrome</b>	6	2, 0,0	0,0,0	1 (0 – 2)	0,0,0

\*expressed as median (range) per grid (high power field x400 magnification)

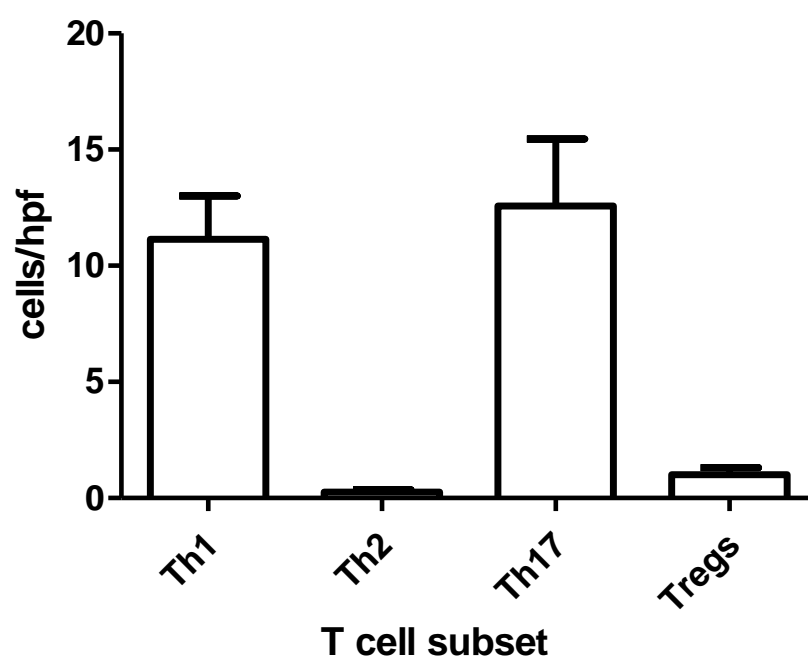
**Table 2: Correlation of Biochemical indices and Th-1 and Th-17 counts.**

	<b>Th-1 (n = 29)</b>	<b>P value</b>	<b>Th-17 (n = 37)</b>	<b>P value</b>
<b>Bilirubin</b>	0.14	0.23	-0.17	0.16
<b>AST</b>	0.07	0.36	0.06	0.47
<b>GGT</b>	-0.1	0.29	0.004	0.49
<b>APRI</b>	<b>0.32</b>	<b>0.05</b>	-0.05	0.36

AST – aspartate aminotransferase

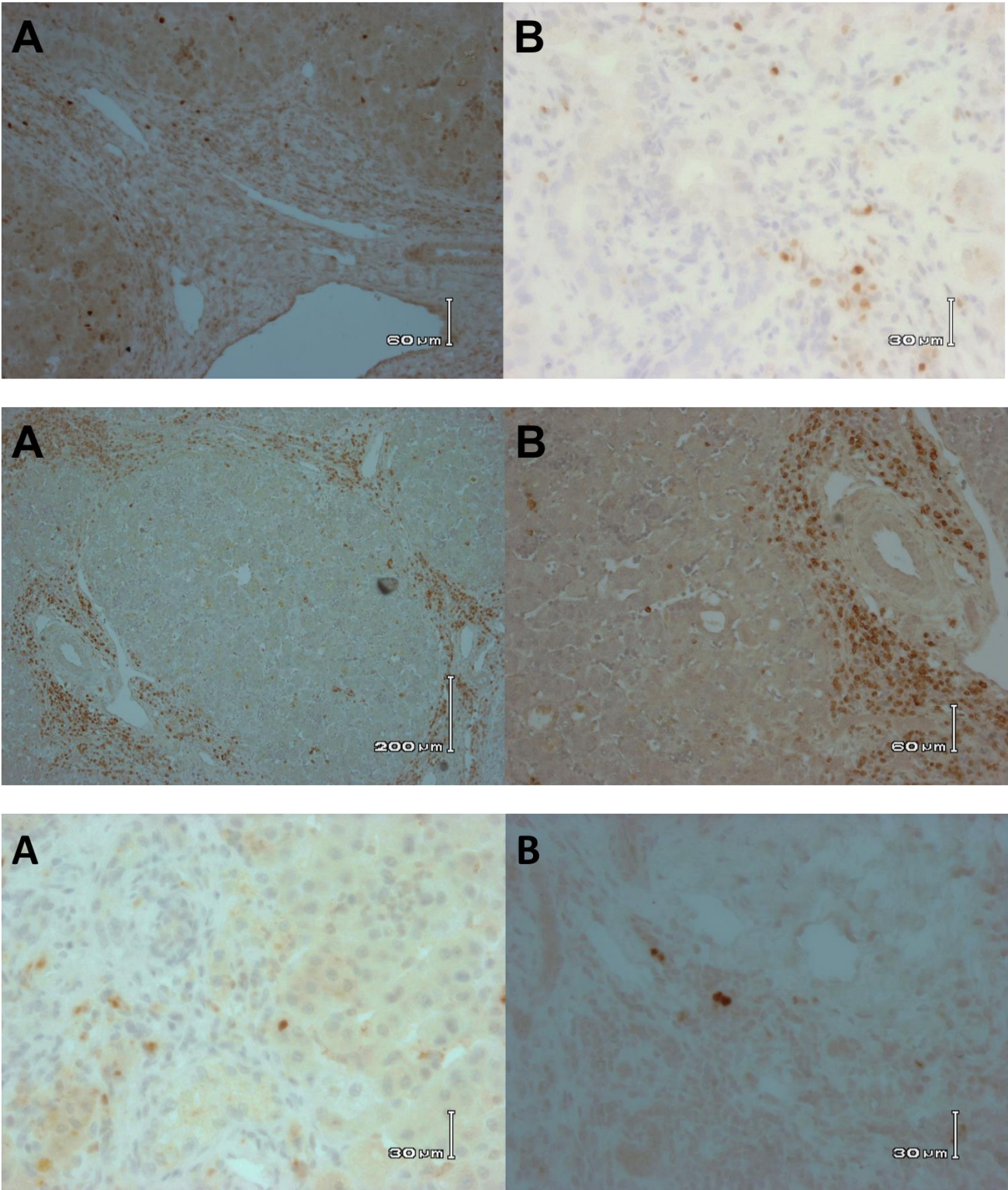
GGT –  $\gamma$ -glutamyl aminotransferase

APRI - Aspartate aminotransferease Platelet Ratio index



**Figure 1:** Quantitation of T cell subsets in biliary atresia (n = 37) (mean +SEM).

Figure 2:



**Legend**

Th-1 (upper), Th-17 (middle ), Treg (lower) and Th-2 (lower) infiltration in infants with biliary atresia.

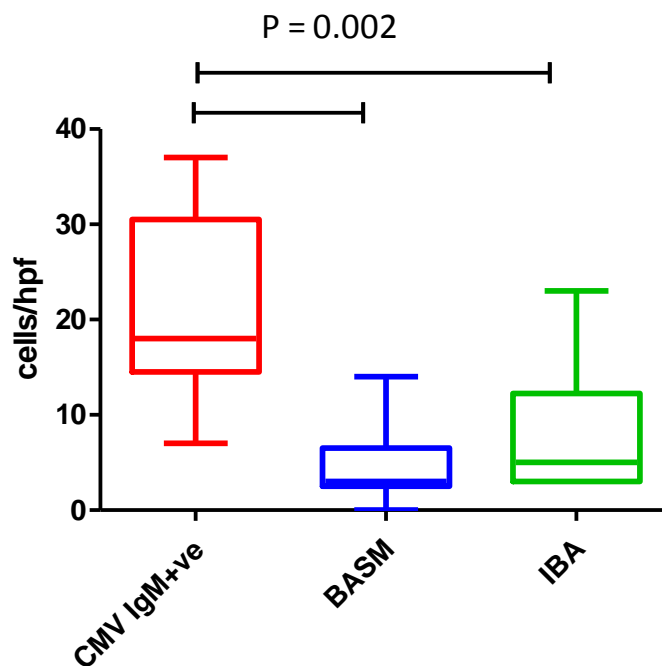
**Upper Row** A (x20) B (x40) Th-1 cells scattered throughout portal tracts principally and also lobule of liver of XX day infant with isolated BA.

**Middle Row** A (x10) B (x20) Th-17 cells heavily concentrated in the portal tracts of a liver from XX day old infant with isolated BA.

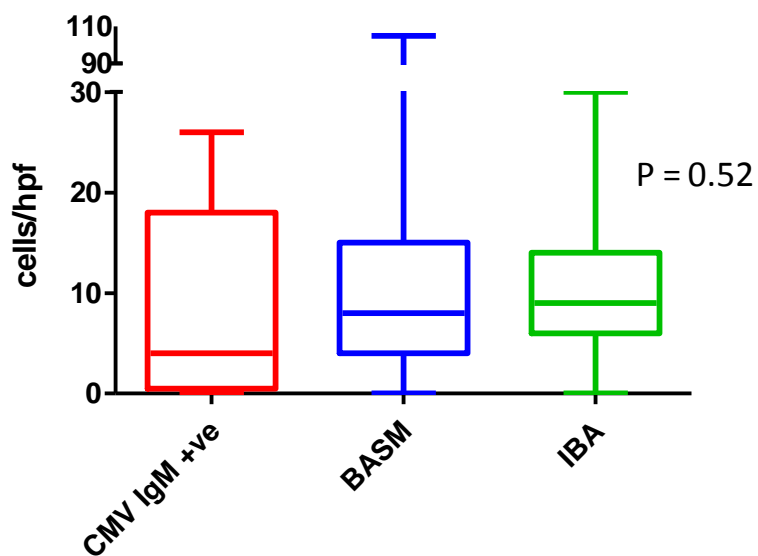
**Lower Row** A (x40) B (x40). **A** Shows very few Treg (FOXP3+) cells in liver from XX day infant with isolated BA. **B** shows very few Th2 cells in a BA patient again at 40x magnification.



FIGURE 3(a)

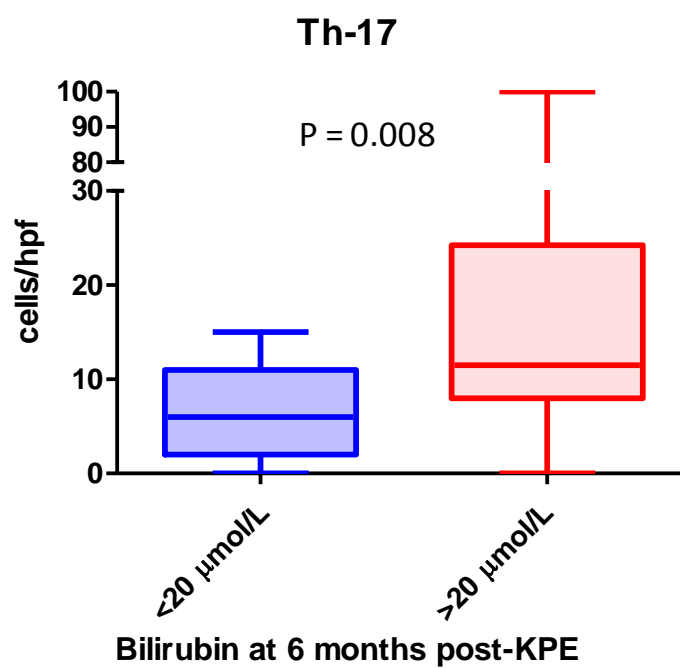


3(b)

**Figures 3a & b:**

T cell subset quantification [(a) Th1 and (b) Th17] according to perceived aetiology: CMV IgM+ve BA (n = 9); BASM (n = 9); isolated BA (n = 19).

Figure 4(a)



4(b)

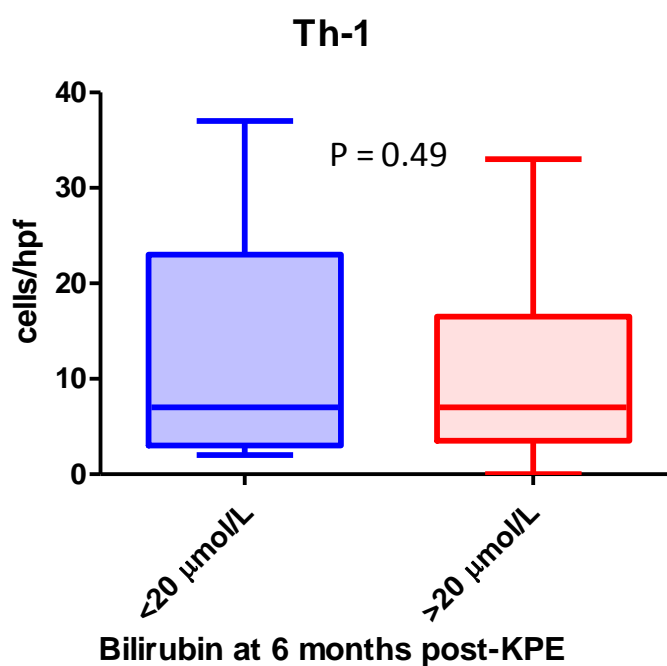
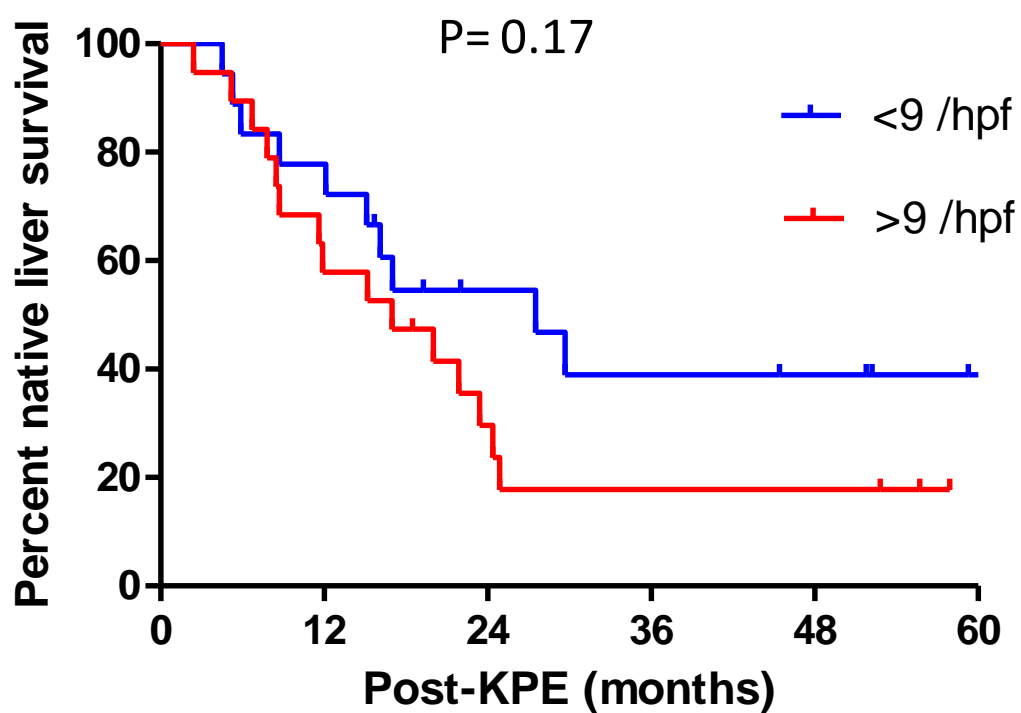


Figure 4 (a &amp; b): Clearance of jaundice and portal tract T cell subset [Th17 (a) and Th1 (b)]



**Figure 5:**  
Native liver survival curve - High ( $\geq 9$  / hpf) versus Low ( $<9$  hpf) Th-17 portal tract infiltration.